

## Original Article

# Regulatory effect of interferon regulatory factor-1 on lymphatic enhancing factor 1

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**Abstract:** Interferon regulatory factor (IRF)-1 is one important transcriptional factor and exerts critical roles in anti-virus and anti-tumor. Lymphatic enhancing factor (LEF)-1 is widely expressed in various tissues, and is significantly up-regulated in tumor tissues/cells, thus is believed to be correlated with tumor occurrence and progression. However, the role of IRF-1 on tumor progression via regulating LEF-1 has not been reported. *In vitro* cultured colon cancer cell line HCT116 was randomly divided into five groups, which were transfected with pcDNA3.1-IRF-1 plasmid, empty plasmid, siRNA-IRF-1, scramble siRNA control, or empty control respectively. Real-time PCR was used to test mRNA level of IRF-1 and LEF1, while LEF1 protein expression was quantified by Western blotting. MTT assay was used to detect proliferation of tumor cells, while Transwell chamber assay was deployed to analyze cell invasion. The activity of Caspase 3 was further measured by test kit. The over-expression of IRF-1 significantly inhibited mRNA and protein expression of LEF1, inhibited tumor cell proliferation, elevated Caspase 3 activity and suppressed tumor invasion ( $P < 0.05$  compared to control group). siRNA interfered with IRF-1 expression and significantly facilitated LEF1 expression, thus potentiating tumor cell proliferation, depressing Caspase 3 activity, and increasing tumor invasion ( $P < 0.05$  compared to control group). IRF-1 can regulate LEF1 expression, as the up-regulation of IRF-1 can inhibit LEF1 expression, further mediating tumor occurrence and progression.

**Keywords:** Interferon regulatory factor-1, lymphatic enhancing factor-1, transfection, tumor

## Introduction

Lymphatic enhancing factor (LEF)-1 is one member of mammalian LEF/T cell cytokine family, and is one lymphatic specific high migration fractional transcriptional factor. Early studies attributed LEF-1 in the participation of thymic cell differentiation [1, 2]. Human LEF-1 gene locates on 4q23~25 region and encodes about 400 amino acids to synthesize two isoforms [2, 3]. As a gene having multiple start codons, a full length protein form containing  $\beta$ -catenin binding domain, or a truncated form without  $\beta$ -catenin binding domain could be generated by the binding on N terminus or alternative site, respectively. Both isoforms can bind or inhibit  $\beta$ -catenin binding, forming or preventing transcriptional coactivators, eventually recruiting or inhibiting  $\beta$ -catenin/LEF complex, thus modulating downstream target gene of Wnt signal pathway [4, 5]. LEF1 is thus believed to be one critical regulatory factor of Wnt signal

pathway. With advancement of research, LEF1 has been confirmed to be correlated with abnormal development of multiple tissues, and can modulate histogenesis [6]. Relatively higher expression level of LEF1 has been found in embryonic cells. After birth, however, LEF1 expression level was suddenly decreased, leading to only trace or even undetectable LEF1 proteins [7, 8]. However, LEF1 expression is remarkably increased when body has tumors, as its elevated expression has also been found in tumor cell line [9, 10].

Interferon regulatory factor (IRF)-1 is one important regulatory factor during interferon biosynthesis and also one critical transcriptional factor. In normal physiological process of cells, it can participate in regulating cell proliferation, cell growth, apoptosis, signal transduction, and anti-virus, anti-tumor or hematopoietic cell development [11, 12]. IRF-1 is widely distributed in various body tissues/organs. Due to its

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important role in cell growth/developmental regulation, IRF-1 can bind with interferon promoter as one intermediate messenger, further regulating other transcriptional factors, thus playing a critical role among multiple pathophysiological mechanisms [13, 14]. The regulatory role of IRF-1 on tumor progression via LEF1, however, is still unknown yet.

### Materials and methods

#### *Reagent and equipment*

Human colon carcinoma cell line HCR116 was purchased from ATCC cell bank (US). DMEM culture medium, fetal bovine serum (FBS) and streptomycin-penicillin were purchased from Hyclone (US). DMSO and MTT powders were purchased from Gibco (US). Trypsin-EDTA lysis buffer was purchased from Sigma (US). Caspase 3 activity assay kit and PVDF membrane were purchased from Pall Life Sciences (US). EDTA was purchased from Hyclone (US). Western blotting reagent was purchased from Beyotime (China). ECL reagent was purchased from Amersham Biosciences (US). Rabbit anti-human LEF1 monoclonal antibody and mouse anti-rabbit horseradish peroxidase (HRP)-conjugated IgG secondary antibody were all purchased from Cell Signaling (US). Lipo2000 transfection reagent was purchased from Life (US). Transwell chamber was purchased from Corning (US). RNA extraction kit and reverse transcription kit were purchased from Axygen (US). Labsystem Version 1.3.1 microplate reader was purchased from Bio-rad (US).

#### *Cell culture and grouping*

HCT116 cells were resuscitated in 37°C waterbath until fully thawing. Cells at log-phase with 2<sup>nd</sup> to 8<sup>th</sup> generation were randomly divided into control group, IRF-1 over expression group with pcDNA3.1-IRF-1 plasmid transfection, empty plasmid group, IRF-1 inhibitor group with siRNA-IRF-1 expression and siRNA negative control group (scramble group).

#### *Transfection of pcDNA-3.1-IRF-1 plasmid*

Trizol reagent was used to extract RNA from HCT116 cells. Reverse transcription was performed according to the manual instruction, using primers specific for IRF-1 (Forward, 5'-TGCGC ACGTT TTGCC CTGAT-3'; Reverse,

5'-TATAC CTTTG CTCTG CTGTC-3') designed by Primer6.0 and synthesized by Sangon (China). Real-time PCR was performed on target genes under the following conditions: 95°C for 2 min, followed by 35 cycles each containing 94°C for 30 s, 60°C for 50 s and 72°C for 35 s. PCR products were purified in agarose gel electrophoresis. Target gene fragment was ligated with pcDNA3.1 vector at 3:1 ratio at 4°C for 16 h. JM109 competent bacteria were prepared in LB plate and LB medium. Cloned plasmid was transformed into competent bacteria. pcDNA3.1-HPV16 E7 plasmid was concentrated and quantified. Colon cancer cells at log phase were collected and adjusted to 3×10<sup>6</sup> per ml. Cells were inoculated into 6-well plate. pcDNA3.1-HPV16 E7 and pcDNA3.1 plasmid was added into 0.2 ml serum-free culture medium for 15 min incubation at room temperature. The mixture of lipo2000 was added into pcDNA3.1-IRF-1 dilutions for 30 min room temperature incubation. Serum was removed from 6-well plate, and PBS was added for rinsing. 1.6 ml serum-free medium was added into each culture system in parallel with empty transfection control group. Cells were then kept in a humidified chamber with 5% CO<sub>2</sub> at 37°C for 6 h, followed by the application of serum-containing medium for further experiments.

#### *Liposome transfection of IRF-1 siRNA*

IRF-1 siRNA (5'-GATAG ACCTA ATGGA TAG-3') or scrambled control oligonucleotide (5'-ACCTA CTTCC GTAAT GT-3') was transfected into HCT116 cells. In brief, cells were cultured in 6-well plate until reaching 70%~80% confluence. IRF-1 siRNA or negative control sequences were mixed with liposome in 200 µl serum-free medium for 15-min room temperature incubation. Lipo2000 reagent was then mixed with IRF-1 siRNA or controlled dilutions for 30-min room temperature incubation. Serum was removed from cells, followed by PBS rinsing and the addition of 1.6 ml serum-free culture medium. Cells were then kept in a humidified chamber with 5% CO<sub>2</sub> at 37°C for 6 h, followed by the application of serum-containing medium in 48 h continuous incubation for further experiments.

#### *MTT assay for cell proliferation*

HCT116 cells at log-phase were seeded into 96-well plate which contained DMEM medium

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**Table 1.** Primer sequence

Gene	Forward primer 5'-3'	Reverse primer 5'-3'
GAPDH	ACCAGGTATCTGCTGGTTG	TAACCATGATGTCAGCGTGGT
IRF-1	ATCATGGTGATGGACCCCG	ATTCGAAGTCGCAGCTGGTC
LEF1	CTTAGTGGTCTCTGTGACGT	TCACCCTCTCACAGCTTGTCGA

with 10% FBS at  $5 \times 10^3$  density. After 24 h incubation, the supernatant was removed. Cells were randomly divided five groups as above-mentioned. 20  $\mu$ l sterile MTT was added into each test well in triplicates after 48 h cell culture. The supernatant was completely removed, with the addition of 150  $\mu$ l DMSO for 10 min vortex until the complete resolving of crystal violet. Absorbance (A) values was measured at 570 nm in a microplate reader to calculate the proliferation rate in each group. Each experiment was repeated for more than three times.

### *Transwell chamber assay for cell invasion*

Following the manual instruction, Transwell chamber was pre-coated using 1:5 50 mg/L Matrigel dilutions on the bottom and upper layer of the membrane, followed by 4°C air-dry. The chamber was placed in a 24-well plate in triplicates for each group. Control cells were cultured in Transwell chamber without Matrigel. After 48 h, PBS was used to rinse Transwell chamber to remove membrane-fixed cells, which were then fixed in cold ethanol and stained by crystal violet. The number of cells at the lower surface of the micro-pore membrane was then counted in triplicates (N=3).

### *Real-time PCR for mRNA expression of IRF-1 and LEF1*

Trizol reagent was used to extract RNA from all groups for cells. Reverse transcription was performed according to the manual instruction, using primers designed by Primer6.0 and synthesized by Invitrogen, Shanghai (China) as shown in **Table 1**. Real-time PCR was performed on target genes under the following conditions: 55°C for 1 min, followed by 35 cycles each containing 92°C for 30 s, 58°C for 45 s and 72°C for 35 s. Data were collected and calculated for CT values of all samples and standards based on fluorescent quantification using GAPDH as the baseline. Standard curve was firstly plotted using CT values of standards, followed by semi-quantitative analysis by  $2^{-\Delta Ct}$  method.

### *Western blotting for LEF1 protein expression*

HCT116 cell proteins were firstly extracted. In brief, RIPA lysis buffer containing proteinase inhibitor was used to lyse cells on ice for 15~30 min, followed by ultrasound rupture (5 s $\times$ 4) and centrifugation (4°C, 10000 g, 15 min). Supernatants were saved and quantified for protein contents, and were stored at -20°C for further Western blotting. Proteins were then separated using 10% SDS-PAGE, and were incubated with anti-LEF1 monoclonal antibody (1:1000 dilution) at 4°C overnight. On the next day, the membrane was rinsed in PBST, and incubated with 1:2000 goat anti-rabbit secondary antibody for 30 min incubation. After PBST rinsing, ECL reagent was used to develop the membrane, which was exposed under X-ray for observing results. Protein imaging analysis system and Quantity One software were used to scan X-ray films for observing band density. Each experiment was repeated for four times (N=4) for further analysis.

### *Caspase 3 activity assay*

Caspase 3 activity in each group of cells was assayed following the manual instruction of test kits. In brief, trypsin was used to digest cells, followed by 600 g centrifugation for 5 min at 4°C. The supernatant was discarded, with the addition of lysis buffer for 15 min iced incubation. The mixture was then centrifuged at 20000 g for 5 min at 4°C, with the addition of 2 mM Ac-DEVD-pNA. OD values were measured at 405 nm wavelength for calculating caspase 3 activity.

### *Statistical analysis*

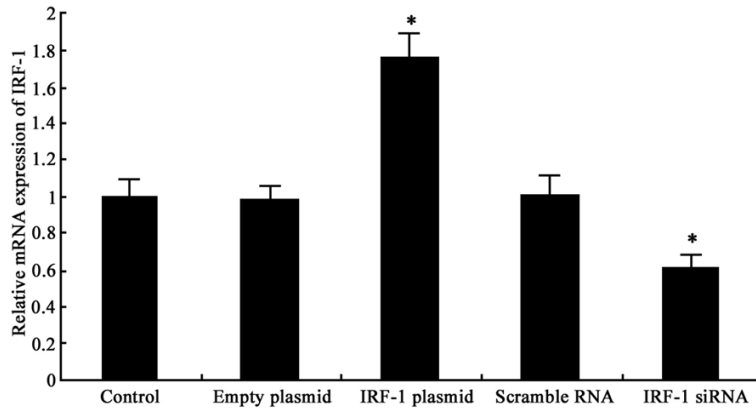
SPSS11.5 software was used in statistical analysis. All data were presented as mean  $\pm$  standard deviation (SD). Student t-test was used to compare means between two groups. Analysis of variance (ANOVA) was used for between-group analysis, followed by LSD test for paired comparison between two groups. A statistical significance was identified when  $P < 0.05$ .

## **Results**

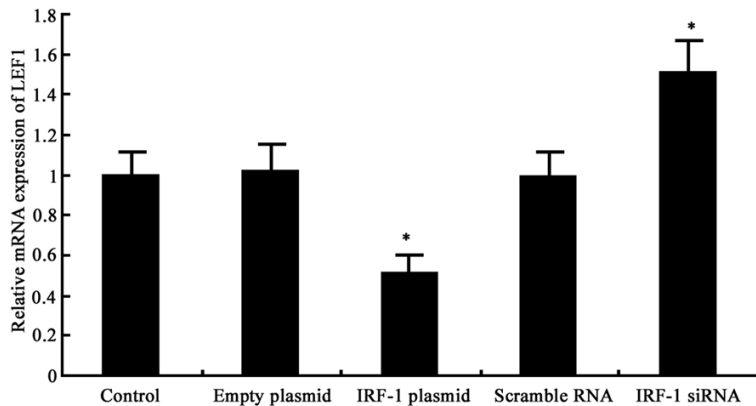
### *IRF-1 expression in HCT116 cells*

We employed real-time PCR to check the effect of IRF-1 vector and siRNA on IRF-1 mRNA

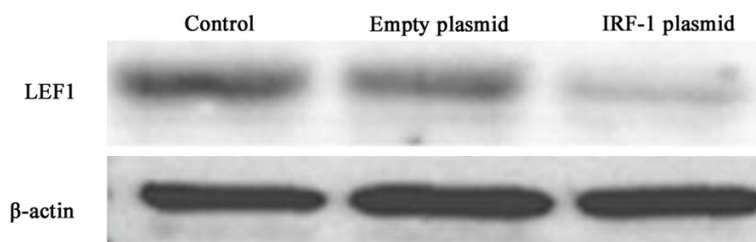
## IRF-1 in tumor progression



**Figure 1.** The effect of IRF-1 manipulation on gene expression in HCT116 cells. \* $P < 0.05$  compared to control group.



**Figure 2.** The effect of IRF-1 manipulation on LEF1 expression in HCT116 cells. \* $P < 0.05$  compared to control group.



**Figure 3.** Over-expression of IRF-1 and LEF1 protein expression.

expression level in colon carcinoma cells. Results showed that the transfection of IRF-1 siRNA in HCT116 cells significantly inhibited the expression of IRF-1 mRNA in HCT116 cells ( $P < 0.05$  compared to control group). The transfection of IRF-1 vector in HCT116 cells significantly facilitated IRF-1 mRNA expression in HCT116 cells ( $P < 0.05$  compared to control group). IRF-1 siRNA negative controlled oligo-

nucleotide or empty plasmid transfection did not affect IRF-1 mRNA expression ( $P > 0.05$  compared to control group, **Figure 1**).

### *Regulatory effect of IRF-1 on LEF1*

Real-time PCR was further used to detect the effect of IRF-1 vector and IRF-1 siRNA transfection on LEF1 mRNA expression level in colon carcinoma cells. Results showed that the transfection of IRF-1 siRNA in HCT116 cells significantly enhanced the expression of LEF1 mRNA in HCT116 cells ( $P < 0.05$  compared to control group). The transfection of IRF-1 vector in HCT116 cells significantly inhibited LEF-1 mRNA expression in HCT116 cells ( $P < 0.05$  compared to control group). IRF-1 siRNA negative controlled oligonucleotide or empty plasmid transfection did not affect LEF-1 mRNA expression ( $P > 0.05$  compared to control group, **Figure 2**).

### *Over-expression of IRF-1 and LEF1 protein expression*

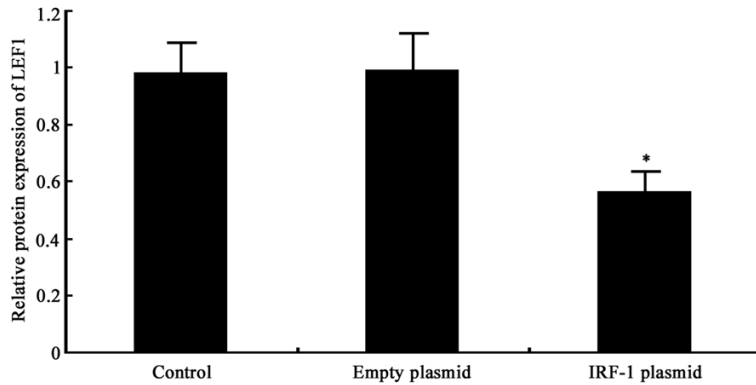
Western blotting was used to test the effect of IRF-1 over-expression on LEF1 protein expression in colon carcinoma cells. Results showed that, similar to those in real-time PCR, the transfection of IRF-1 vector in HCT116 cells significantly inhibited LEF1 protein expression ( $P < 0.05$  compared to control group).

The transfection of empty plasmid did not affect LEF1 protein expression ( $P > 0.05$  compared to control group, **Figures 3 and 4**).

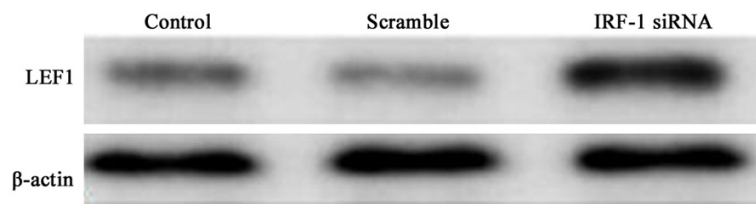
### *Regulatory effect of IRF-1 siRNA on LEF1 protein expression*

Western blotting was further used to test the effect of IRF-1 siRNA transfection on LEF1 pro-

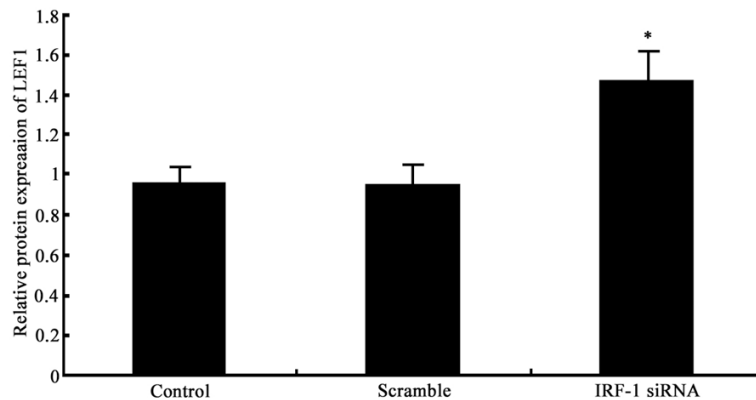
## IRF-1 in tumor progression



**Figure 4.** Analysis of IRF-1 regulation on LEF1 protein expression. \* $P < 0.05$  compared to control group.



**Figure 5.** Gene silencing of IRF-1 and LEF1 protein expression.



**Figure 6.** Analysis of IRF-1 gene silencing on LEF1 protein expression. \* $P < 0.05$  compared to control group.

tein expression in colon carcinoma cells. Results showed that, similar to those in real-time PCR, the transfection of IRF-1 siRNA in HCT116 cells significantly facilitated LEF1 protein expression ( $P < 0.05$  compared to control group). The transfection of siRNA negative control did not affect LEF1 protein expression ( $P > 0.05$  compared to control group, **Figures 5 and 6**).

### *The role of IRF-1 in regulating tumor cell proliferation via modulating LEF1*

MTT assay was used to detect the effect of IRF-1 on tumor cell proliferation via mediating

LEF1. Results showed that after transfecting pcDNA3.1-IRF-1 plasmid, which caused IRF-1 over-expression and LEF1 down-regulation, the proliferation of tumor cells was significantly inhibited ( $P < 0.05$  compared to control group). siRNA interference on IRF-1 expression, on the other hand, significantly potentiated LEF1 expression and facilitated tumor cell proliferation ( $P < 0.05$  compared to control group, **Figure 7**).

### *Caspase 3 activity in tumor cells regulated by IRF-1*

Caspase 3 activity assay kit was used to detect the effect of IRF-1 regulation of LEF1 on caspase 3 activity in tumor cells. Results showed that IRF-1 over-expression significantly inhibited LEF1 expression and elevated caspase 3 activity ( $P < 0.05$  compared to control group). The interference of IRF-1 by siRNA remarkably potentiated LEF1 expression and suppressed Caspase 3 activity ( $P < 0.05$  compared to control group, **Figure 8**).

### *Effect of IRF-1 in regulating tumor cell invasion*

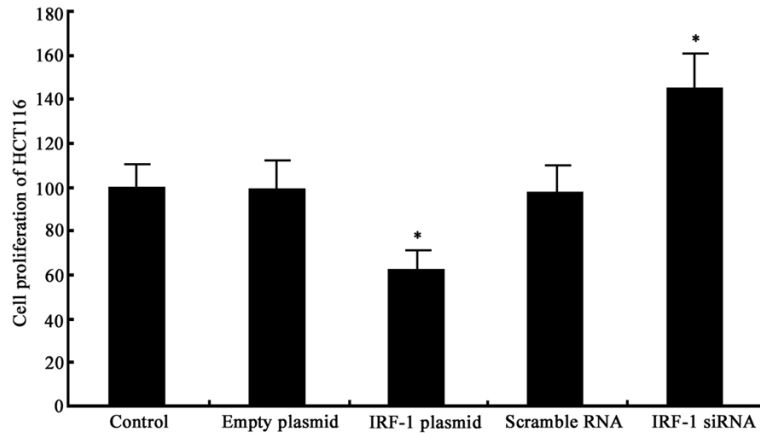
Transwell chamber assay was employed to detect the effect of IRF-1 manipulation on LEF1

expression and tumor cell invasion. Results showed that IRF-1 over-expression inhibited LEF1 expression and suppressed tumor cell invasion potency ( $P < 0.05$  compared to control group). The interference of IRF-1 by siRNA remarkably potentiated LEF1 expression and enhanced tumor cell invasion ( $P < 0.05$  compared to control group, **Figures 9 and 10**).

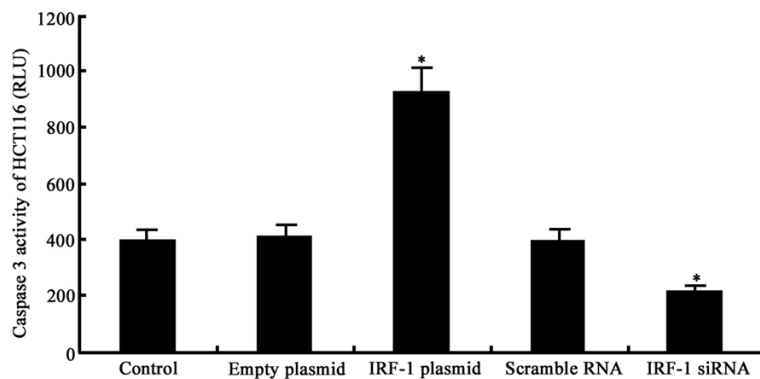
## Discussion

The incidence of tumors is increasing worldwide, and severely affects public healthy and people life quality due to its worse survival rate

## IRF-1 in tumor progression



**Figure 7.** Effect of IRF-1 on tumor cell proliferation. \*P<0.05 compared to control group.



**Figure 8.** The effect of IRF-1 on regulating LEF1 and Caspase 3 activity in tumor cells. \*P<0.05 compared to control group.

and prognosis [15, 16]. Therefore the illustration of tumor pathogenesis and searching for treatment target are always research focuses in medicine. LEF1 is one transcriptional factor and participates in Wnt signal transduction regulation. LEF1 recruits  $\beta$ -catenin, further activating downstream genes, thus becoming one major branch of Wnt signal pathway, which is widely accepted as one major reason for tumor occurrence once being disrupted. Under normal condition, Wnt signal pathway is maintained at silent status. Once its open, LEF1 recruits  $\beta$ -catenin, and activates their interaction, for regulating target genes and activating signal pathways, further leading to abnormal proliferation or activation of cells, inducing tumor occurrence [17, 18]. LEF1 up-regulation can be detected in both colorectal carcinoma tissues and cultured cell lines, with its full length form. Therefore, LEF1 plays a critical role

for tumor proliferation, apoptosis and invasion/metastasis.

IRF-1 has multiple biological functions especially its anti-tumor potency. This study transfected cultured tumor cells with pcDNA3.1-IRF-1 plasmid or siRNA to establish both over-expression and silencing model of IRF in tumor cells. Results demonstrated that the over-expression of IRF-1 significantly inhibited LEF1 mRNA/protein expression, while siRNA-IRF-1 significantly facilitated LEF1 expression, suggesting that IRF-1 could mediate interferon system, and inhibit cell growth/proliferation signals via mediating transcriptional factors including LEF1 [13, 14]. The up-regulation of LEF1 was shown to be correlated with occurrence and metastasis of colorectal carcinoma, and can induce the epithelial mesenchymal transition of tumor cells [19]. IRF-1 can enhance Caspase 3 activity and inhibit tumor invasion via mediating LEF1 to suppress

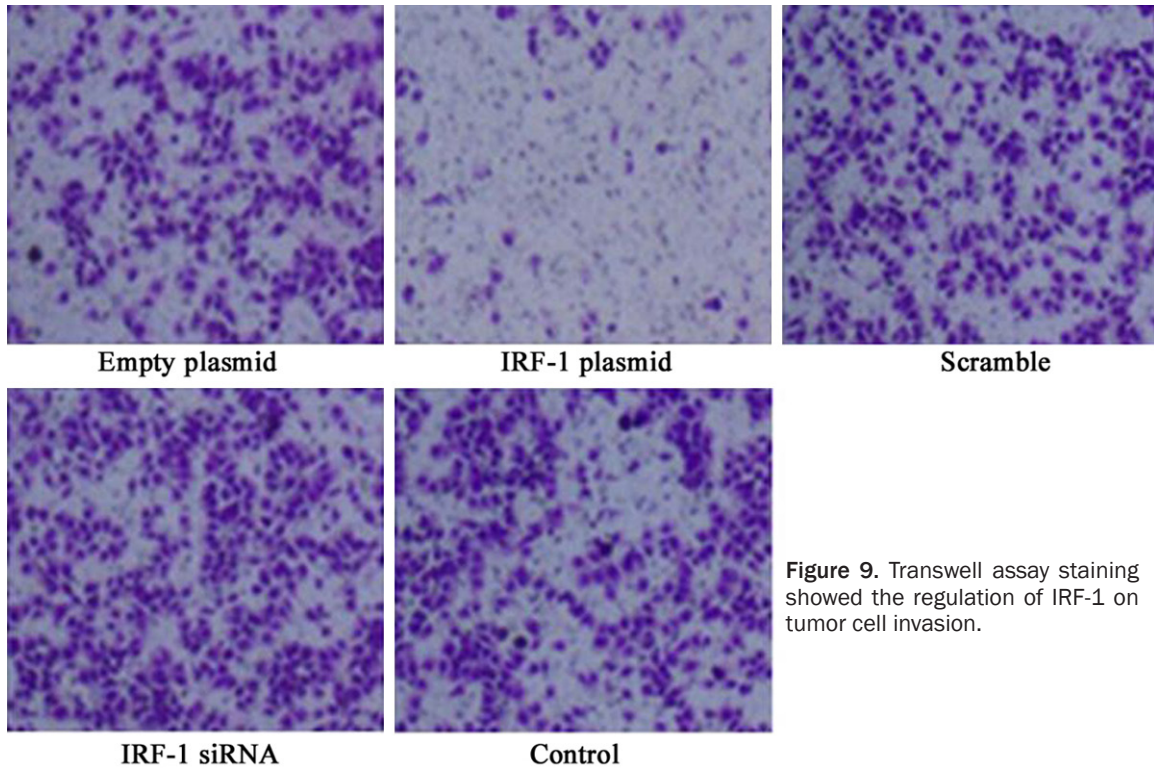
tumor cell proliferation. IRF-1 has a wide spectrum of expressions. It exerts its roles via binding with interferon promoter, and on other transcriptional factors. IRF-1 induced cysteine proteinase expression [20]. Therefore this study demonstrated that IRF-1 can affect tumor progression via mediating LEF1.

In conclusion, IRF-1 can regulate LEF1, as the up-regulation of IRF-1 can inhibit LEF1 expression, further affecting tumor occurrence and progression.

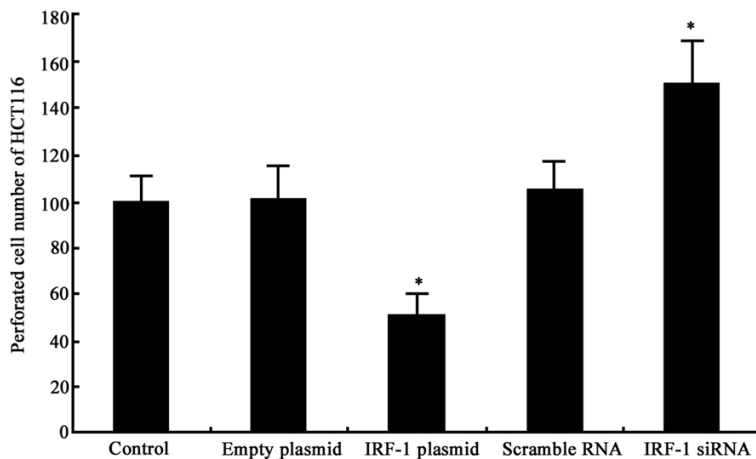
### Acknowledgements

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## IRF-1 in tumor progression



**Figure 9.** Transwell assay staining showed the regulation of IRF-1 on tumor cell invasion.



**Figure 10.** Analysis of IRF-1 effect on tumor cell invasion. \* $P < 0.05$  compared to control group.

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### Disclosure of conflict of interest

None.

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